MOLECULAR AND CELLULAR BIOLOGY, Nov. 1989, p. 5254–5259
0270-7306/89/115254-06$02.00/0

Vol. 9, No. 11

RNA Polymerase II Transcription Termination Is Mediated Specifically by Protein Binding to a CCAAT Box Sequence

SHEILA CONNELLY† AND JAMES L. MANLEY*

Department of Biological Sciences, Columbia University, New York New York, 10027

Received 6 June 1989/Accepted 10 August 1989

A region in the adenovirus major late promoter (MLP) containing a CCAAT consensus sequence can direct transcription termination of RNA polymerase II, a mechanism that possibly prevents transcriptional interference from upstream genes. Using a chimeric plasmid template that contains the MLP directing expression of the simian virus 40 early region, we showed that an inserted oligonucleotide containing only 13 base pairs of MLP sequences, including the CCAAT box, is capable of inducing transcription termination in an orientation-dependent, position-independent manner. Point mutations within the CCAAT-specific protein-binding site abolished this effect, while a base substitution outside of this region did not affect termination. These data suggest that termination is mediated by a CCAAT box-binding protein. Several other transcription factor-binding sites do not, however, cause termination, suggesting that this may be a relatively specific property of a CCAAT-binding protein.

The mechanisms responsible for transcription termination of mRNA-encoding genes in eucaryotic cells are poorly understood. Several recent studies have shown that efficient termination of transcription by RNA polymerase II (pol II) requires a functional poly(A) addition site (8, 22, 25, 36). However, transcription continues well beyond this site before terminating (for reviews, see references 4 and 31), and another signal that delineates the region or site at which termination occurs is necessary to direct efficient termination (9, 25). We have shown that a sequence containing an inverted CCAAT box consensus in the adenovirus major late promoter (MLP) functions as such a termination site signal (9). Since a number of proteins that bind CCAAT box sequences have been recently identified (6, 7, 10, 20, 26, 32) and, more specifically, a protein purified from HeLa cells, CP1, has been shown to bind to the CCAAT sequence within the MLP (20), it is possible that protein binding to the MLP CCAAT box mediates termination induced by this sequence. However, the terminator regions analyzed previously all contained MLP sequences in addition to the CCAAT consensus. Furthermore, the ability of the fragments to induce termination was orientation dependent, perhaps suggesting that a function other than protein binding is involved.

To gain insights into the mechanism by which this termination site functions, we have investigated the ability of a short oligonucleotide containing either the CCAAT consensus or mutant derivatives to elicit termination when inserted downstream of a poly(A) site. For these studies, pφ4-SVA, a chimeric plasmid that consists of the MLP (Ad2 nucleotides [nt] −405 to +33) directing transcription of the simian virus 40 (SV40) early region (SV40 nt 5171-2533) in a pBR322 vector (Fig. 1A) (24), was used to direct transcription in transient expression assays. The plasmid pSV2.CAT (12) was cotransfected in all cases to standardize transfection efficiencies (8, 9). Assays were performed using human 293 cells (1, 13), which have been shown to support high levels of transcription from pφ4-SVA (24). We showed previously that two DNA fragments, one containing MLP sequences from −125 to −73 relative to the transcription start sites, and the other containing sequences from −85 to −51, were both capable of inducing termination when inserted into the *Sal*I site (pBR322 nt 652) of pφ4-SVA located 330 base pairs (bp) downstream from the SV40 poly(A) site (9). These results together suggested that MLP sequences between −85 and −73 may be sufficient to induce termination.

To test this hypothesis directly, a 13-bp oligonucleotide containing this sequence was inserted into the *Sal*I site of pφ4-SVA (Fig. 1B). Plasmids containing single insertions in either the orientation in which this sequence is naturally found within the MLP, wt(1+), or in the opposite orientation, wt(1−), were identified and transfected into 293 cells. Forty-eight hours later, nuclei were isolated and analyzed in nuclear run-on assays (31). These assays measure the number of polymerase molecules actively engaged in transcription of the DNA template at the time of nuclei isolation. Nascent RNA chains were elongated in the presence of [α-^{32}P]GTP for 10 min exactly as described previously (9). RNA was extracted from the transfected cell nuclei and hybridized to a consecutive series of DNA restriction fragments extending downstream from and including the poly(A) site (Fig. 1C). If the inserted oligonucleotide were capable of directing termination, then a reduction in the amount of RNA hybridized to fragments downstream from this site (within fragment E, Fig. 1C) would be detected. The results of this analysis are shown in Fig. 2. In both the wild-type and wt(1−) samples, similar amounts of RNA hybridized to fragments D through I. The wt(1+) sample, although it showed a similar level of RNA hybridized to fragment D, showed reduced amounts of RNA hybridized to fragments F through I. The amount of RNA hybridized to each fragment was quantitated and standardized by liquid scintillation counting of each slot cut from the nitrocellulose filters. The termination rate, computed as described previously (9), was calculated from the decrease in the transcription rate between fragments upstream of the potential termination site and fragments downstream from this site. The wt(1+) sample showed an approximate 78% termination rate, compared with 0% for wt or wt(1−) samples. These results directly indicate that the CCAAT box sequence is, by itself, capable of inducing termination in an orientation-dependent manner.

* Corresponding author.
† Present address: Friedrich Miescher-Institut, Basel, Switzerland, CH-4002.

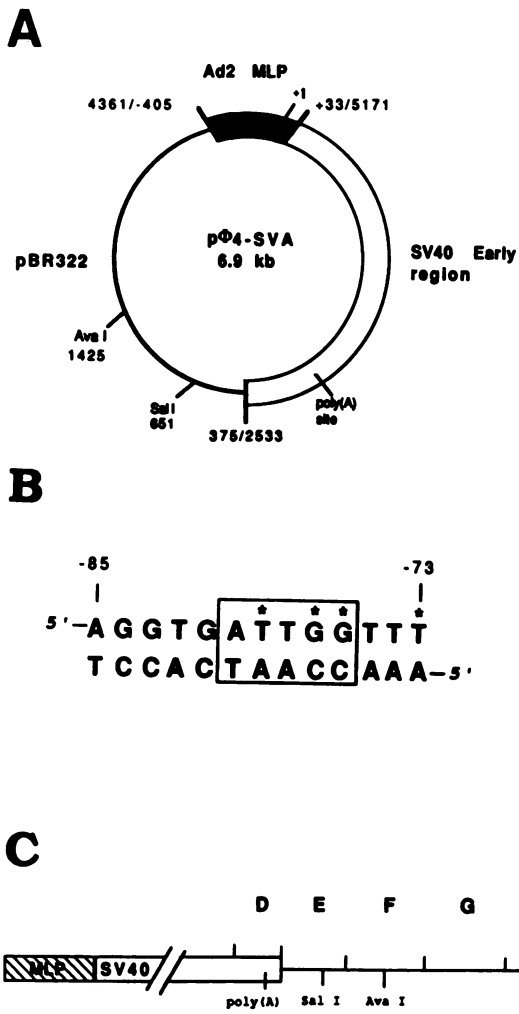

FIG. 1. Map of pϕ4-SVA, oligonucleotide insertion, and probes used for nuclear run-on analysis. (A) Plasmid pϕ4-SVA contains adenovirus MLP sequences from −405 to +33 controlling expression of the SV40 early region (SV40 nt 5171 to 2533) within a pBR322 vector (pBR322 nt 375 to 4363). (B) The 13-bp oligonucleotide contains MLP sequences from −85 to −73 with respect to the transcription initiation site (+1). The inverted CCAAT sequence is boxed, and mutated bases are indicated (*). The oligonucleotide was inserted into the *Sal*I site (pBR322 nt 651) or the *Ava*I site (pBR322 nt 1425) of pϕ4-SVA or derivatives that had been filled in with the Klenow fragment of DNA polymerase. The orientations of the inserts were determined by restriction enzyme analysis, and point mutations were identified by sequence analysis as described previously (35). (C) A schematic diagram showing the pϕ4-SVA-derived restriction fragments used in nuclear run-on experiments. Fragments D through I extend from the 3′ end of the SV40 early region, through pBR322, and up to the MLP and have been described previously (9).

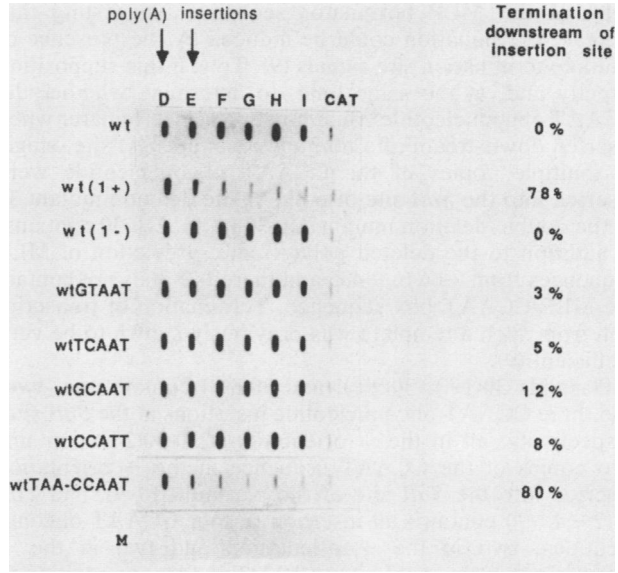

FIG. 2. Nuclear run-on analysis of wild-type and mutant oligonucleotide insertion plasmids. Cells were transfected with the indicated plasmids, and nascent RNA chains were elongated in the presence of [α-^{32}P]GTP. Labeled RNA (3×10^7 cpm) was hybridized to the indicated pϕ4-SVA-derived restriction fragments (D through I) or to a chloramphenicol acetyltransferase (CAT) gene-specific restriction fragment isolated from pSV2.CAT, which was used to quantitate transfection efficiencies. Each fragment was bound to the nitrocellulose slots in a molar amount equivalent to 4 μg of pϕ4-SVA DNA (vertical rows). Horizontal rows show separate filters hybridized to ^{32}P-labeled RNA elongated in nuclei isolated from cells transfected with the indicated plasmid or with no plasmid (M). The termination rate downstream from the insertion site (fragment E) was calculated, and the result, presented as a percentage, is shown beside each filter.

If termination is due to protein binding to the CCAAT consensus, then base substitutions that interfere with this binding should inactivate the terminator, while mutations elsewhere in the 13-bp fragment should not. To test this, three single-base changes and one double mutation were introduced into the CCAAT sequence, and one single-base change was introduced outside the consensus (the asterisks in Fig. 1B denote the positions mutated). Plasmids containing insertions in the (+) orientation were identified, and the resulting mutants, wtGTAAT, wtTCAAT, wtGCAAT, wtCATT, and wtTAA-CCAAT, which are named according to the sequence of the lower strand in Fig. 1B, were transfected and analyzed by nuclear run-on assays. The results, shown in Fig. 2, revealed that all of the oligonucleotide insertions that contain point mutations within the CCAAT sequence did not induce termination, as judged by the approximately equal hybridization to fragments upstream and downstream of the insertion site. However, a reduction in the amount of RNA hybridized to fragments downstream of the wtTAA-CCAAT insertion was detected. Termination efficiency was calculated as 80%, comparable with the termination efficiency of wt(1+). wtTAA-CCAAT contains an A-to-T transversion at MLP nt −73, which is outside the CP1 protein-binding site consensus sequence (6). Gel retention assays using purified CP1 protein and DNA fragments containing CCAAT elements isolated from a variety of promoters have shown that high-affinity CP1 binding occurs with promoters that contain any of the four possible bases at this site, suggesting that this nucleotide is not important for protein binding (6). Because mutations within the protein-binding site abolish termination and a mutation outside of it does not affect termination efficiency, these data provide strong evidence that protein binding at the MLP CCAAT sequence mediates transcription termination by RNA polymerase II.

We showed previously that deletion of the SV40 early poly(A) site in pϕ4-SVA resulted in an increase in the transcription rate of mutant templates because of a decrease in termination efficiency (8). Termination could, however, be partially restored on such templates by insertion of a second

copy of the MLP terminator sequence, suggesting that increased termination could be induced by the presence of multiple termination site signals (9). To test this supposition directly and, at the same time, to determine whether the CCAAT oligonucleotide could function as a terminator when inserted downstream of a nonfunctional poly(A) site, single or multiple copies of the CCAAT oligonucleotide were inserted into the *Sal*I site of poly(A) site deletion mutant 30 or the double-deletion mutant 210-30 (8, 9). 210-30 contains, in addition to the deleted poly(A) site, a deletion of MLP sequences from −66 to −405 and therefore does not contain the MLP CCAAT box sequence. Termination of transcription from such a template was previously shown to be very inefficient (9).

Plasmids 30(1+), 30(2+), and 30(3+) contain one, two, and three CCAAT oligonucleotide insertions at the *Sal*I site, respectively, all in the + orientation. 210-30(2+) contains two copies of the CCAAT sequence in the + orientation inserted into the *Sal*I site of the plasmid 210-30, and 210-30(2+,4+−) contains an insertion of four CCAAT oligonucleotides, two in the + orientation and two in the − orientation, in the *Ava*I site (pBR322 nt 1425) in addition to two copies inserted in the *Sal*I site. These plasmids were transfected into 293 cells, and the nascent transcripts produced were analyzed in nuclear run-on assays (Fig. 3A). All samples from templates containing CCAAT oligonucleotide insertions showed reductions in the amount of RNA hybridized to fragment D upstream from the insertion site compared with 30 and 210-30 samples, indicating that the CCAAT oligonucleotide is capable of directing termination on plasmid templates lacking a functional poly(A) site. Furthermore, quantitation by liquid scintillation counting of [^{32}P]RNA hybridized to fragments upstream and downstream of the inserted CCAAT box revealed that termination occurred at or near this sequence. As expected, the calculated termination efficiencies (~30 to 50%) were significantly lower than in the presence of a functional poly(A) addition site. Insertion of tandem copies of the CCAAT oligonucleotide did not appreciably increase termination efficiency [compare 30(1+), 30(2+), and 30(3+) samples]. An increase in termination efficiency was observed, however, in a comparison of 210-30(2+) and 210-30(2+,4+−) samples. The calculated termination rates for 210-30(2+) and 210-30(2+,4+−) were 30 and 52%, respectively, indicating that multiple CCAAT sequences do increase termination efficiency when inserted more than 700 bp apart. Why do separated CCAAT sequences result in increased termination, while adjacent sequences do not? An explanation for this may be that in plasmids containing tandem inserts [i.e., 30(3+)], all three CCAAT sequences are not simultaneously available for protein binding, possibly because of steric constraints. When CCAAT sequences are located at different sites within the template, such as within the promoter and at the *Sal*I site [compare 30(1+) and 210-30(2+)] or inserted at the *Sal*I and *Ava*I sites [compare 210-30(2+,4+−) and 210-30(2+)], all of these sequences are available for protein binding and can thus function to increase the overall termination efficiency.

Since protein binding at the MLP CCAAT sequence is capable of inducing termination, perhaps other transcription factor-binding sites have a similar function. We showed previously that MLP sequences from −66 to −51, which encompass the MLP upstream factor-binding site (USF/MLTF; 5, 34), do not induce termination. However, it has been shown that the dissociation rate of USF is decreased upon simultaneous binding of TFIID to the TATA sequence

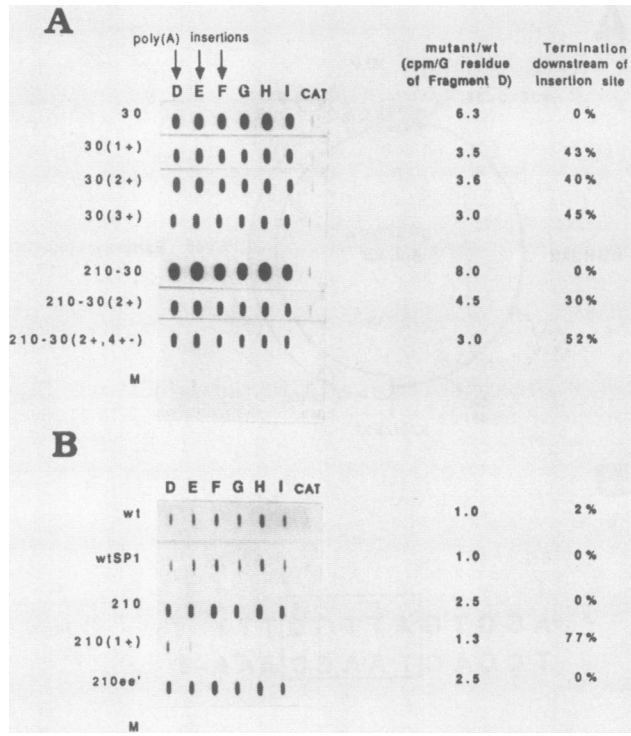

FIG. 3. Nuclear run-on analysis of additional insertion mutant plasmids. (A) Multiple CCAAT insertions. (B) Other transcription factor-binding site insertions. Nuclei were isolated from cells transfected with the indicated DNAs, and nascent RNA chains were labeled as described in the text. Labeled RNA (3×10^7 cpm) was hybridized to nitrocellulose-bound restriction fragments D through I isolated from pϕ4-SVA and the CAT fragment as described in the text. Horizontal rows show separate filters hybridized to RNA isolated from cells transfected with the indicated plasmids or with no plasmid (M). The transcription rates of the mutants relative to pϕ4-SVA (wt) were calculated as shown and are displayed to the right of the filters, as are the calculated termination efficiencies at the insertion sites in fragments E and F.

and USF to its binding site and vice versa, suggesting that these two proteins functionally interact (34). Therefore, USF binding may not be stable enough to induce termination when its binding site alone is inserted into pϕ4-SVA. We have shown previously that termination of transcription by RNA pol II can occur within the SV40 promoter/origin region during in vitro transcription (14). This region contains multiple transcription factor-binding sites, including six tandem SP1-binding sites (for a review, see reference 27). For this reason, we analyzed the SV40 SP1-binding sites (21-bp repeats) for possible termination function. A *Fok*I (SV40 nt 80)-*Sty*I (SV40 nt 37) fragment, which contains five of the six SP1 recognition sites, was isolated from SV40 DNA and inserted into the *Sal*I site of pϕ4-SVA in the orientation in which these sites are found within the SV40 genome, thus creating wtSP1. Because multiple factors have also been shown to bind specifically to the SV40 enhancer region (for a review, see reference 21), p210-SVAee′ (38), a plasmid containing two copies of the SV40 enhancer element (72-bp repeats) inserted in opposite orientations into the *Sal*I site of MLP deletion mutant p210-SVA (23), was also analyzed. Previous studies have shown that the presence of the SV40 enhancer sequence has no effect on transcription initiation from plasmid templates transfected into 293 cells (24). Nu-

clear run-on experiments were performed with nuclei isolated from cells transfected with pϕ4-SVA (wt), wtSP1, p210-SVA (210), p210-SVA containing the wild-type CCAAT oligonucleotide insert [i.e., 210(1+)], and p210-SVAee′ (210ee′). The results of this analysis are shown in Fig. 3B. Approximately equal amounts of RNA hybridized to fragments D through I were detected with wt, wtSP1, 210, and 210ee′ samples. Because we have shown that the CCAAT oligonucleotide directs termination when inserted into a wild-type plasmid template (Fig. 2) and when inserted into a mutant template that lacks a CCAAT sequence in the MLP [210(1+), Fig. 3], these results indicate that the SP1-binding sites and the SV40 enhancer sequences do not induce termination.

If the SV40 enhancer is indeed unable to block transcription elongation in vivo, then its presence in pϕ4-SVA should not affect the dramatic accumulation of nuclear RNA that occurs as a result of multiple rounds of transcription in cells transfected with mutants containing inactive poly(A) sites (8). As mentioned above, the CCAAT box-containing terminator fragment is able to block such accumulation (9). To test the effect of the enhancer on accumulation of nuclear RNA, derivatives of pϕ4-SVA containing enhancer sequences as well as poly(A) site deletions were analyzed. Plasmids pϕ4-SVAe, 6e, 12e, 15e, and 16e, constructed by inserting the *Bst*XI (SV40 nt 4759)-*Bam*HI (SV40 nt 2533) fragment of mutants 6, 12, 15, and 16 (8), which contain the poly(A) site deletions, into the *Bst*XI-*Bam*HI site of pϕ4-SVAe, were analyzed. pϕ4-SVAe is identical to pϕ4-SVA except that the former contains the SV40 72-bp repeats inserted at the *Eco*RI site (pBR322 nt 4361), approximately 400 bp upstream of the MLP (24). Nuclear RNA was isolated from cells transfected with the indicated plasmids and subjected to quantitative S1 analysis. The amount of RNA used in each reaction (30 μg or less) was standardized for any variation in transfection efficiency and hybridized to a 3′ end-labeled DNA probe that spans the pre-mRNA poly(A) site and digested with S1 nuclease exactly as described previously (8). The results of this analysis are shown in Fig. 4. RNAs properly cleaved at the wild-type poly(A) site generate a 230-nt protected fragment, as seen with pϕ4-SVAe (e) and 6e. We have shown previously that the deletion in mutant 6 does not affect polyadenylation (8). The larger protected fragments represent unprocessed RNA, the sizes of which are dependent on the endpoints of the deletion in each plasmid, i.e., the point at which the transcript and DNA probe are no longer homologous. A substantial accumulation of nuclear RNA was detected in cells transfected with mutants 12e, 15e, and 16e, which have deleted sequences known to be necessary for efficient 3′ end formation (8). The amount of nuclear RNA produced by the poly(A) site deletion mutants (10- to 20-fold more than the wild type) is similar to the amount detected in identical studies using pϕ4-SVA without the enhancer sequence (8). This analysis, therefore, indicates that the enhancer sequences do not reduce the accumulation of steady-state nuclear RNA and confirms the results of the nuclear run-on analysis.

We have presented data to suggest that protein binding to the CCAAT box region of the MLP mediates transcription termination induced by this sequence. How might protein binding induce termination? It has been suggested previously that sites that elicit the termination event are polymerase pause sites (for a review, see reference 31). The stalling of the polymerase at such pause sites could destabilize the transcription complex and in this way directly induce termination, or it could allow a *rho*-like termination factor to catch

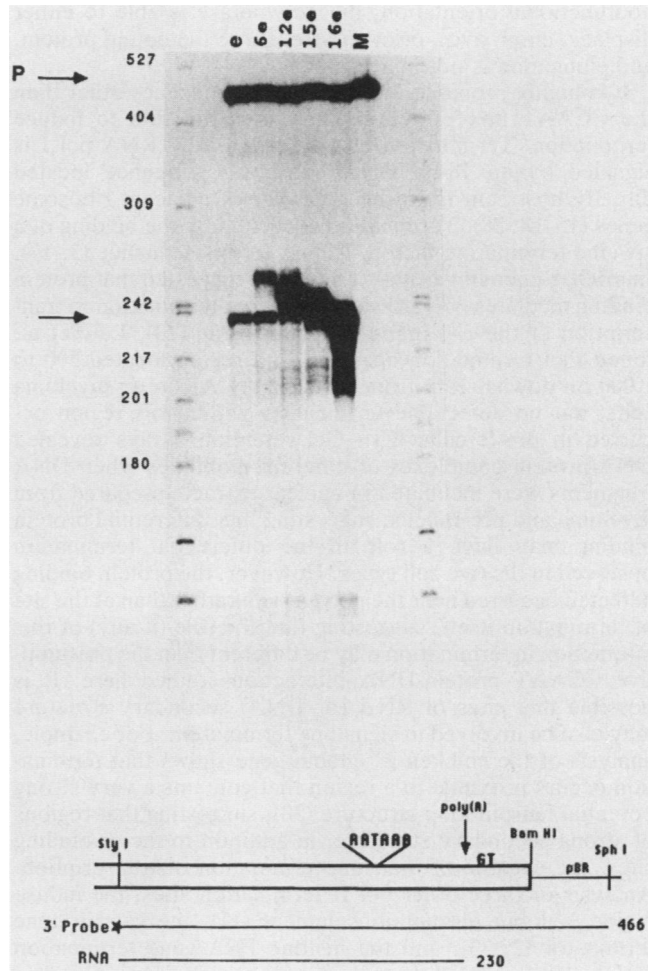

FIG. 4. Analysis of steady-state nuclear RNA from pϕ4-SVAe- and poly(A) site mutant-transfected cells. Nuclear RNA was isolated from cells transfected with the indicated plasmids and analyzed by S1 nuclease protection. RNA 3′ ends were mapped using a 3′-end-labeled DNA probe isolated from pϕ4-SVA extending from the SV40 *Sty*I site (nt 2812) to the pBR322 *Sph*I site (nt 562). The *Bam*HI site demarks the boundary between SV40 and pBR322 sequences. S1 nuclease reactions were carried out exactly as described previously (8). Arrows mark the S1 nuclease-protected fragments and the undigested probe (P). Size marker (lane M) is pBR322 digested with *Hpa*II and 5′ end labeled. Numbers at left indicate size (in nucleotides). The structures of the probe and S1 nuclease digestion products are indicated.

up to the polymerase, resulting in termination (9). However, the termination caused by protein binding to the CCAAT box is probably more specific than simply a barrier to elongation to the transcribing polymerase. We have shown that a variety of other transcription factor-binding sites do not elicit termination and that the CCAAT oligonucleotide functions in only one orientation. An explanation for this may be that the transcription complex, or a component thereof, specifically interacts with the CCAAT protein bound at this site. It appears that at least several CCAAT-binding proteins are heterodimeric (6, 7), and it is possible that the protein that binds to the MLP CCAAT consensus (CP1) possesses two functionally different surfaces, only one of which is able to interact with and block the transcribing polymerase. When the CCAAT sequence is present in the

nonfunctional orientation, the polymerase is able to either displace, climb over, or otherwise ignore the bound protein, and elongation is not inhibited.

It is highly probable, however, that sequences other than the CCAAT box protein-binding sites function to induce termination. Termination of transcription by RNA pol I is signaled by an 18-bp DNA consensus sequence located directly upstream from the promoter of adjacent ribosome genes (15–18, 28). Termination is elicited by the binding of a specific termination factor, TTF-1, to this sequence (3, 17). In mRNA-encoding genes, it has been suggested that protein binding mediates poly(A) site-dependent termination in transcription of the μ-δ immunoglobulin gene (23). Law et al. found that termination occurred in a region located 500 to 1,000 bp downstream from the μ_m poly(A) site in myeloma cells, but no detectable termination within this region occurred in pre-B cells (23). Gel retention assays revealed DNA-protein complexes of different mobilities when DNA fragments were incubated in nuclear extracts prepared from myeloma and pre-B cells, suggesting that differential protein binding may have a role in the differential termination observed in the two cell types. However, the protein binding detected occurred near the poly(A) site rather than at the site of termination itself, suggesting that the role (if any) of this interaction in termination may be different than the presumptive CCAAT protein-DNA interaction studied here. It is possible that sites of RNA (or DNA) secondary structure may also be involved in signalling termination. For example, analysis of the chicken β^H-globin gene shows that termination occurs proximal to a region that contains a very strong potential hairpin loop structure (30), suggesting that regions of strong secondary structure, in addition to factor-binding sites, are capable of inducing termination of transcription. Analysis of three other pol II termination sites, the mouse major β-globin termination element (11), the gastrin gene terminator (2, 33), and the histone H2A gene termination region (19), revealed that all are composed of long stretches of AT-rich sequences. Whether these regions contain factor-binding sites and/or regions of secondary structure remains to be determined. Indeed, the SV40 promoter/origin, where termination has been shown to occur in vitro (14), contains, in addition to the protein-binding sites mentioned above, several potential RNA hairpin loop structures. Since we have shown here that these binding sites do not elicit termination, it is possible that the secondary structure in this region is the termination signal. It will be interesting to determine directly whether this region or other regions of secondary structures are capable of inducing termination when inserted into the plasmid templates described in this study.

We have presented evidence indicating that the presence of a CCAAT box-binding site can induce less efficient termination in the absence of a poly(A) site. Perhaps in this situation the polymerase is not released from the DNA, a question that is difficult to address by nuclear run-on assays. It is interesting to speculate, however, that this is relevant to the mechanism by which premature termination occurs. Premature termination of transcription occurs shortly after initiation in a number of genes, many of which appear to be subject to regulation (for a review, see reference 31). These observations together suggest that control of transcriptional termination may be as widespread a mechanism of gene regulation in eucaryotes as it is in procaryotes (for reviews, see references 29 and 37). Characterization of the actual mechanism of poly(A) site-dependent transcription termination and premature termination by RNA pol II and the *trans*-acting factors involved in this process must await the advent of soluble in vitro systems capable of reproducing these reactions.

We thank R. Stewart and L. Lee for excellent technical assistance and T. Coudreaut for preparing the manuscript.

This work was supported by Public Health Service grant GM 28983 from the National Institutes of Health.

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